AUG 29 1932

VOLUME III NUMBER 2

STAIN TECHNOLOGY

APRIL, 1928

CONTENTS

Progress in the Standardization of Stains.
Stain Substitutes. H. J. CONN 41

The Spectrophotometric Evaluation of Mixtures of Methylene Blue and Trimethyl Thionin. WALTER C. HOLMES 45

The Effect of Preliminary Treatment (Fixing Fluids) on Staining Properties of the Tissues. ALEXANDER V. TOLSTOOUHOV 49

Staining Cell Constituents in Diseased Plant Tissues. J. DUFRENOY 57

Further Studies on a Modification of the Gram Stain. NICHOLAS KOPELOFF and PETER COHEN 64

Discussion of Paper on "Metallic Lakes of the Oxazines as Nuclear Stain Substitutes for Hematoxylin." PERCY A. HOUSEMAN 70

Notes on Technic.
A New Substitute for Ethyl Alcohol in the Gram Stain. H. J. CONN 71

Published by the

COMMISSION ON STANDARDIZATION OF BIOLOGICAL STAINS

Entered as second-class matter at the Post Office, Geneva, N. Y.

STAIN TECHNOLOGY

ISSUED QUARTERLY SUBSCRIPTION PRICE $1.50 PER YEAR

Editor and Business Manager
H. J. CONN
Soil Bacteriologist, N. Y. Agr. Exp. Station

Consulting Editors
CHEMISTRY: W. C. HOLMES Color Lab., U. S. Dept. of Agriculture
ZOOLOGY: S. I. KORNHAUSER School of Medicine, Univ. of Louisville
PATHOLOGY: F. B. MALLORY Boston City Hospital
BOTANY: W. R. TAYLOR Univ. of Pennsylvania

SUGGESTIONS TO AUTHORS SUBMITTING MANUSCRIPT

Address all correspondence regarding manuscripts for publication, proofs, etc. to H. J. Conn, Agr. Exp. Station, Geneva, N. Y. Articles to be accepted for publication must ordinarily deal with the nature or uses of biological stains. Articles dealing with microscopic technic in general, however, will be accepted in most cases, if they have a close bearing on staining procedures. Brief notes concerning new staining methods are especially solicited. It is understood that articles submitted for publication have not appeared previously elsewhere and that they will not be offered for simultaneous appearance in other publications without the consent of the editor of STAIN TECHNOLOGY.

To be accepted for publication, manuscript should be original typewritten copy (not carbon copy) either double or triple spaced, with wide margins. Special attention should be given to the headings of tables and to legends for illustrations. These in all cases should be complete in themselves, so as to make the table intelligible to anyone studying it, without consulting the text of the article. In the case of complicated tables, typewritten manuscript is not absolutely required, provided all words and figures are carefully written or printed in a legible hand. All references to literature must be carefully verified and in ordinary cases the complete title and reference should be given.

Articles over two printed pages in length (i. e. about 800 words) must be preceded by a brief summary (100–400 words) which can be used as an author's abstract by abstracting journals. This summary must therefore be complete in itself, and must contain all the important facts brought out in the paper.

All illustrations should accompany the manuscript and should be numbered, with the legend for each written on separate paper and attached loosely to the illustration. They should always be referred to in the text. Line drawings must be made in India ink. Photographs for half-tones must be on paper giving black and white tones and should preferably have a glossy finish.

REPRINTS

Reprints of each article will be furnished at approximately the cost of printing. Orders for reprints and covers should be sent to the editor immediately upon receipt of proof of the article.

STAIN TECHNOLOGY

VOLUME III APRIL, 1928 NUMBER 2

PROGRESS IN THE STANDARDIZATION OF STAINS

STAIN SUBSTITUTES

The early biologists naturally used for stains such dyes as were readily available to them on the market. In many instances their choice has not proved the best possible for the future development of microscopy. The natural dyes are difficult to obtain in constant quality. Some of the early anilin dyes were mixtures not easily duplicated today; others, difficult to manufacture on a commercial scale, have since been replaced by more easily made dyes, so that the original product is now obsolete commercially. Still others were unsatisfactory in some respect both for staining and for textile dyeing, often because the colors produced were not fast. No blame, of course, attaches to the early biologists for the use of such dyes, as they employed to the best of their ability those that were available to them. Today, however, the use of dyes chosen by early microscopists is sometimes awkward. Frequently, a dye now obsolete for textile purposes, such as thionin, has to be made especially for microscopic use, and sometimes its manufacture is quite expensive. Recent research, therefore, has been directed toward the possibility of securing substitutes in some such instances as these. The present article is intended to call attention to some of these efforts.

GENTIAN VIOLET

Gentian violet is an instance where one of the early dyes was a mixture of unknown composition. In this case the name was one not even used in textile dye industry, the most similar dye being called methyl violet. Biologists, nevertheless, even to this day, continue to demand gentian violet; and as a result, stain companies are obliged to sell something labeled gentian violet even tho the commercial name of the dye may be something else.

The realization of this confusion lead to the investigations, reported elsewhere,[1] to show whether gentian violet could be better replaced by crystal violet or by one of the methyl violets. The results, as shown before in these pages,[2] have been to indicate that for most

[1]H. J. Conn, et al. An investigation of American gentian violets. Report of Com. on Bacteriological Technic. J. Bact. 7, 529–536. 1922.

H. J. Conn. Biological Stains, Geneva, N. Y., 1925. See p. 68.

[2]H. J. Conn. Grades of gentian violet. Stain Techn. 1, 53–55. 1926

purposes crystal violet is the best substitute, but that where a redder shade is desired one of the methyl violets, such as the shade denoted 2B, may be employed.

PYRONIN

Before the war the pyronin furnished biologists was labeled pyronin G, and it was generally supposed that this grade was necessary. When the domestic companies were first trying to produce stains for biologists, it was pointed out that pyronin G is expensive and difficult to prepare, as it is made from an intermediate which is not, or at least was not then, produced in this country; while pyronin B is easily manufactured, is very similar chemically, and is almost the same shade as pyronin G. This dye was tried out, and as has been reported before[3] is an entirely satisfactory substitute for the dye used before the war. American stain companies now regularly supply this grade instead of pyronin G. It is, of course, correctly labelled "pyronin B" on the bottles.

MAGDALA RED

Magdala red is necessarily an expensive dye; and the substitution of a cheaper product was begun in Germany, either intentionally or unintentionally, before the war. There were two grades of "magdala red" supplied biologists; one was labeled "magdala rot," the other "magdala rot, echt." It seems, as reported before,[4] that only the latter was correctly labeled. The former stain was not magdala red in any sense of the term, but was an entirely different dye, an acid dye of the eosin group instead of a basic dye of the safranin group; it was apparently either erythrosin or phloxine. Recently Chamberlain[5] has shown that phloxine gives the same results as the so-called "magdala rot" of Grübler, in staining algae. He finds, further, that both the true magdala red manufactured in this country and "magdala rot, echt" Grübler give unsatisfactory results in the technic in which he is accustomed to employ the stain.

HEMATOXYLIN

Hematoxylin is a natural dye, the composition of which is known to vary according to the methods used in its manufacture. For this reason Proescher and Arkush have recently[6] proposed a series of synthetic dyes one or more of which they hope may replace hema-

[3]H. J. Conn. Biological Stains. p. 76.

[4]Id. p. 57.

[5]C. J. Chamberlain. Staining with phloxine. Stain Techn. **2**, 91–93. 1927.

[6]F. Proescher and A. S. Arkush. Metallic lakes of the oxazines (Gallamin blue, gallocyanin and Coelestin blue) as nuclear stain substitutes for hematoxylin. Stain Techn. **3**, 28–38. 1928.

toxylin. The value of some of these dyes, as indicated by the staining reactions described in their paper, can hardly be questioned; but whether they will prove substitutes for hematoxylin or will rather add to the list of stains available for securing varied staining effects, remains for the future to decide.

It should be remarked, however, that there never was a time when there was less need of a substitute for this valuable dye than now. The present product is more constant than ever before and seems to prove generally satisfactory. This fact is emphasized in an article later in this issue.[7] While that remains the case, it is unlikely that biologists familiar with the use of hematoxylin will experiment with substitutes with which they are unfamiliar. Nevertheless, the work of Proescher and Arkush must be regarded as a very valuable contribution to the art of staining.

LIGHT GREEN

Various dyes, better known by other names, are sometimes called light green commercially, such as malachite green and methyl green; but when the name is used by biologists, however, it seems generally to refer to the dye more correctly called light green, SF yellowish. This dye has valuable properties as a cytoplasmic stain, but is unfortunately very likely to fade. A recent paper by Haynes[8] shows that fast green FCF[9] is a very satisfactory substitute for this dye and fades very slowly, if at all.

In connection with this newer dye, it is very interesting to add a note as to its color. The dye is green in acid solutions and blue in alkaline, having noticeable indicator properties. At pH 6 the dye exists practically wholly in the green form; at pH 7 it contains about 6% of the blue form; at pH 10 it is almost wholly in the blue form. Solutions of this dye contain equal proportions of both forms at pH 8.15; in other words its dissociation constant is close to that of phenol red and its sensitive range lies slightly to the alkaline side of neutrality. Its value as an indicator is not yet known; but its color change must be taken into account in using it as a stain. Miss Haynes, for instance, finds the dye bluer than light green SF yellowish; but it is entirely possible that someone using it in a different technic, in which the reaction of the staining solution or of the material stained is more acid, might find the shade distinctly yellower.

[7]Houseman, P. Discussion of paper on Metallic lakes of the oxazines as nuclear stain substitutes for hematoxylin. Stain Techn. 3, 71. 1928.

[8]Haynes, Rachel. Fast Green, a subs'itute for light S. F. Yellowish. Stain Techn., 3, 40. 1928.

[9]Manufactured by the Warner-Jenkins Co. of St. Louis.

Whether the possibility of obtaining more than one shade with this dye adds to its value as a stain or not, remains for the future to determine.

H. J. CONN

THE SPECTROPHOTOMETRIC EVALUATION OF MIXTURES OF METHYLENE BLUE AND TRIMETHYL THIONIN

WALTER C. HOLMES

(Contribution No. 148 from the Color and Farm Waste Division, Bureau of Chemistry and Soils, U. S. Department of Agriculture, Washington, D. C.)

ABSTRACT.—**Spectrophotometric analysis affords the most convenient means for determining the proportion of methylene blue and trimethyl thionin (azure B) present in a mixture of these two dyes. The method proposed depends upon the determination of an "absorption ratio." A suitable ratio for the purpose is that of the extinction coefficient at 640 mμ to that at 670 mμ. On account of the difference in absorption maxima of the two dyes, this ratio increases as the percentage of methylene blue decreases. The ratio value for eleven different mixtures is given and a graph is plotted from this data by means of which the proportions of the two dyes present in any mixture can be calculated from the absorption ratio determined as specified.**

Recent investigations of the penetration of dyes into living cells have emphasized the need of reliable methods for distinguishing between methylene blue and trimethyl thionin (Azure B) in biological solutions and for determining the relative proportions in which the dyes are present. Spectrophotometric analysis affords the most convenient means for this purpose.

The principal absorption maximum of methylene blue in dilute aqueous solutions is at approximately 665 mμ, and the corresponding maximum of trimethyl thionin is at approximately 650 mμ. The determination of the approximate absorption maximum, accordingly, differentiates aqueous solutions of the individual dyes with absolute certainty. It affords, moreover, a reliable criterion of the gross character of solutions in which both dyes are present. Owing to the relatively limited spectral interval between the bands of the individual dyes, the absorption of a mixture does not exhibit the individual maxima of its components but, rather, affords a single composite band with a maximum of which the spectral location varies with the relative proportions of the dyes. The absorption maximum is displaced in the direction of shorter wave length in direct proportion with increasing proportions of trimethyl thionin in the dye mixture.

Whereas, however, the location of the absorption maxima is adequate in establishing the essential character of the dye content of

solutions, the differentiation afforded is only crudely quantitative. In view of the average low visual sensitivity in the spectral region in question and other considerations, it appears probable that a variation of 10 or 15% in dye proportions may be required to afford a displacement in the absorption maximum which may be recognized with certainty.

A far more satisfactory means for differentiating between the dyes in question is found in the employment of an "absorption ratio." A suitable ratio for the purpose is that of the extinction coefficient at 640 mμ to the extinction coefficient at 670 mμ. The wave lengths selected lie on the opposite slopes of both absorption bands, thereby insuring maximum alteration in ratio value with unit variation in

TABLE 1. ABSORPTION RATIOS OF MIXTURES OF METHYLENE BLUE AND TRIMETHYL THIONIN.

Dye Sample (*approximate*)	Ratio Values†	
	47.5% Alcohol	50% Acetic Acid
90.0% M. B., 10% T. M. T*	0.73 (±.01)	0.76 (±.01)
80.0% M. B., 20% T. M. T.	0.80 "	0.83 "
70.3% M. B., 29.7% T. M. T.	0.88 "	0.90 "
60.8% M. B., 39.2% T. M. T.	0.95 "	0.98 "
51.6% M. B., 48.4% T. M. T.	1.05 "	1.08 "
42.5% M. B., 57.5% T. M. T.	1.15 "	1.18 "
33.6% M. B., 66.4% T. M. T.	1.26 "	1.29 "
24.9% M. B., 75.1% T. M. T.	1.37 "	1.405 "
16.5% M. B., 83.5% T. M. T.	1.52 "	1.56 "
8.1% M. B., 91.9% T. M. T.	1.69 "	1.73 "
100.0% T. M. T.	1.90 "	1.94 "

*In this table M. B. stands for methylene blue and T. M. T. for trimethyl thionin. These figures have been corrected to agree with composition of the dyes as explained in *supplementary note.*

†These values are the ratios between the extinction coefficients of the mixtures at 640 mμ to the corresponding coefficients at 670 mμ.

the relative proportions of the dyes. The character of the dyes in question renders it advisable to modify the nature of the solvent in which the ratio is measured. In aqueous solution the absorption of both dyes is influenced in a decided degree by variation in dye concentration and in a minor degree by variation in neutral salt concentration, and both dyes also exhibit a tendency toward absorption of dye base on the surface of containing cells, which may interfere, in a minor degree, with accurate measurements of light absorption. The effect of these factors is negligible in 50% alcohol or 50% acetic acid, and it is recommended, accordingly, that the aqueous dye

solution under examination be diluted with an equal volume of 95% alcohol or an equal volume of glacial acetic acid.

In order to obtain the basic data necessary the dyes were synthesized from intermediates of the best grade obtainable. Methylene blue was prepared in accordance with the directions given by Fierz-David and was subsequently recrystallized from water. Trimethyl thionin was synthesized by the same method by means of substituting a molar equivalent of monomethyl anilin for the dimethyl anilin in the oxidation with dimethyl-p-phenylenediamine thiosulphonic acid. Stock solutions of both dyes were prepared in distilled water, and their content of actual dye was determined by means of titration with titanous chloride. Dilute aqueous solutions were prepared from these containing known proportions of the dyes, the total dye content ranging from 5 to 9 milligrams of dye per liter. The ratio of extinction-coefficients referred to was determined with these solutions after further dilution (a) with an equal volume of 95% alcohol and (b) an equal volume of acetic acid. A 2 c.m. absorption cell was used. The measurements were carried out with a Hilger wave-length spectrometer equipped with a Nutting photometer.

The experimental values are given in Table 1; and the data thus obtained are plotted in the accompanying graph.

The sensitivity of the method varies somewhat, increasing with increasing proportions of trimethyl thionin in the dye mixture. The average alteration in the value of R. is approximately 0.01 for each per cent of trimethyl thionin in the mixture. It appears probable that under favorable conditions of dye concentration a variation of 2% in the proportion of trimethyl thionin in the dye mixture can be detected with but little uncertainty.

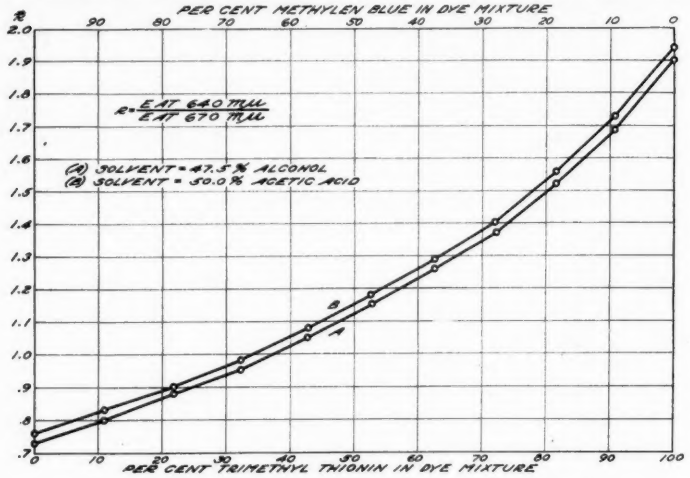
Further investigation has indicated that moderate variations in hydrogen ion concentration, dye concentration, and neutral salt concentration are without appreciable influence upon the ratios and that the method may be relied upon over a wide range of conditions.

SUPPLEMENTARY NOTE

Before the measurements recorded in the foregoing paper were undertaken the methylene blue sample employed was compared with several samples of the medicinal grade of the dye which were available. All samples gave approximate checks. Their absorption ratios by the acetic acid technic ranged between 0.75 and 0.76. Altho it was known that all of the dyes in question contained trimethyl thionin, it seemed probable, in view of this agreement, that the amount of the latter dye present was small and its effect upon the absorption of the dye slight. It was decided, however, to attempt

the further purification of the dye, and a suitable method of purification was found subsequently.

One gram of methylene blue was dissolved in a liter of distilled water and 100 ccm. of twentieth molar borate buffer solution added. The combined solutions were shaken vigorously for fifteen minutes with 250 cc. of chloroform. This chloroform extraction was subsequently repeated with fresh solvent thirty times. At the end of this treatment it was evident that further extraction would be ineffective. The methylene blue solution still contained trimethyl thionin which could not be removed. It was obvious, however, that the proportion of residual trimethyl thionin was very small. The absorption ratio of


PER CENT METHYLEN BLUE IN DYE MIXTURE
90 80 70 60 50 40 30 20 10 0
2.0 1.9 1.8 1.7 1.6 1.5 1.4 1.3 1.2 1.1 1.0 .9 .8 .7
R = EAT 640 mμ / EAT 670 mμ
(A) SOLVENT = 47.5 % ALCOHOL
(B) SOLVENT = 50.0 % ACETIC ACID
B
A
0 10 20 30 40 50 60 70 80 90 100
PER CENT TRIMETHYL THIONIN IN DYE MIXTURE

GRAPH 1. ABSORPTION RATIOS OF MIXTURES OF METHYLENE BLUE AND TRI-METHYL THIONIN.

Dye proportions in this graph not corrected. To correct them the values indicated for methylene blue must be multiplied by 0.9. See *supplementary note.*

the purified methylene blue was found to be 0.67 by the alcohol technic and 0.69 by the acetic acid technic. These values indicate the removal of approximately 10% of trimethyl thionin from the dye solution.

It is obvious, accordingly, that the methylene blue employed in standardizing the method was not a substantially pure sample, as was supposed, but contained approximately 10% of trimethyl thionin. The data recorded in Table 1 have been corrected to agree with these findings but those in Graph 1 require a corresponding revision. This may be done by multiplying the values for percent of methylene blue by 0.9.

THE EFFECT OF PRELIMINARY TREATMENT (FIXING FLUIDS) ON STAINING PROPERTIES OF THE TISSUES

ALEXANDER V. TOLSTOOUHOV

Sydenham Hospital Laboratory, New York City

ABSTRACT.—**As was reported in a previous paper,[1] staining properties depend on the chemical composition of the tissues and on the strength of the dyes themselves. Applying mixtures of basic and acid dye on tissues (methylene blue, eosin Y) at different pH-values, it is possible to find differences in the isoelectric points of the nuclei and cytoplasm of different tissues. For example, the nucleus of polymorphonuclear cells of the blood consists of the most acid protein, with an isoelectric point around pH 2.5, while the nucleus of lymphatic tissues has an isoelectric point of about pH 4.0, and that of connective tissue about pH 3.4.**

With a knowledge of the above, a constant method of staining at various pH-values was used to study the effect of different fixing fluids on the staining properties of the tissues. In this way it was found that many fixing fluids gave very stable compounds with tissue proteins, and that they almost permanently change the chemical composition (i.e. the staining properties of the tissues). In some instances, these changes can be easily explained from the regular chemical standpoint. For example, formalin forms inert compounds with amino groups of the amino acids of proteins and in this way it makes the tissue proteins more acid, i.e. it moves the isoelectric point of the proteins toward a lower pH-value. The same is true in the case of the polivalent acids. The bivalent heavy metals such as mercury, on the contrary, it is assumed, combine with carboxyl groups of amino acids and in this way move the isoelectric point of the proteins toward a higher pH.

As follows from the writer's previous studies,[1] the staining properties of the tissues depend mostly on three factors: (1) the strength of the dye; (2) the strength of the tissue proteins or the isoelectric points of the proteins; and (3) the pH-value of the dye solutions.

Applying mixtures of a basic and an acid dye (methylene blue medicinal Merck, and eosin Y) of constant composition but of varying reaction, it was possible to stain differentially any kind of tissues, including the bacteria. It was shown that staining at different pH-values, with a mixture of a basic and an acid dye is not only a

[1]Tolstoouhov, A. V. Some Practical Applications of the Physico-chemical Theory of Differential Staining: Blood, Tissue, and Bacteria Staining. Methylene blue eosin water-soluble Mixtures as an Universal Dye Mixture. Proc. N. Y. Path. Soc. N. S. **26**, 147–159. 1927.

method of differential staining, but is also a method for determining the isoelectric points of the tissue proteins. In this article, the sensitiveness of the above mentioned method is shown, and the effect of fixing fluids on the staining properties of the tissues.

To study the sensitiveness of the method, a series of slides from the same block of fixed tissue were taken and stained at different pH-values with the mixture of methylene blue medicinal Merck plus eosin Y, in the manner described in my previous paper.

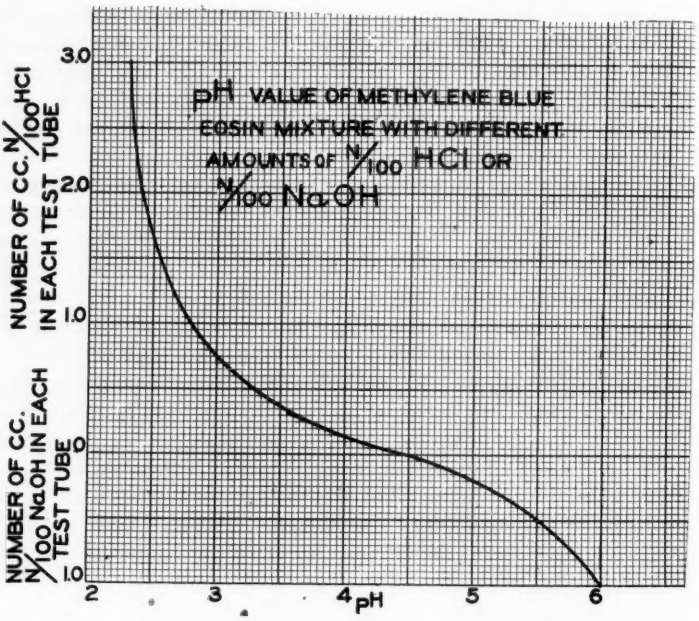


CHART 1. REACTION OF THE EOSIN-METHYLENE-BLUE MIXTURE WITH DIFFERENT AMOUNTS OF N/100 HCl OR N/10 NaOH IN EACH TEST TUBE OF 10 C.C. Determinations of pH-value made with a DeEds electrode.

The methylene blue plus eosin mixture at various pH-values may be made up as follows: Prepare separately in two bottles, 0.15% methylene blue medicinal and 0.5 eosin Y (or W. S., i. e. water soluble) in pure methyl alcohol. Mix the solutions together in the proportions of 15 c.c. eosin to 85 c.c. methylene blue. Shake. This mixture is stable. To adjust the mixture to various pH-values: Pipette into a series of test tubes either $\frac{N}{100}$ NaOH or $\frac{N}{100}$ HCl, into the first tube 0, in the second 0.2 c.c., into the third 0.4 c.c.; then successively 0.6, 0.8, 1.0, 2.0, 3.0 c.c. To each tube add distilled water to 5 c.c. mark. Then add 5 c.c. of the above described dye mixture. Shake and stain. These dilutions of the dye mixture with HCl or NaOH are not as

stable, and for staining they should be made fresh. The pH-value of each test tube can be found from Chart 1. This chart is prepared from data already published by the author.[2]

The results of one of these analyses are presented in Chart 2. As seen from this chart: at about pH 2.8 everything is stained by eosin except the nuclei of the polymorphonuclear cells of the blood, which are stained by methylene blue. At about pH 3.4, the nuclei of the connective tissue begin to be stained by methylene blue. These of the lymphatic tissue, composing the tumor itself, are stained by methylene blue at a still more alkaline reaction, about pH 4.0.

From these findings, in accordance with the writer's previous studies, we conclude that the nuclei of the different tissues differ in chemical composition. The most acid is the nucleoprotein of the polymorphonuclear cells, with an isoelectric point around pH 2.5; the most alkaline is the nucleoprotein of the lymphatic tissue tumor cells, with an isoelectric point around pH 4.0. In a similar way an analysis of the cytoplasm of different cells can be made. To do this we should examine the chart, beginning with the highest pH and proceeding toward the lower pH-values.

Studying in the same way the staining properties of different organs of the same animal, fixed in the same fluid, we were able to find differences in the chemical composition of the different organs. At the present time we are studying the difference in the staining properties of one group of bacteria.[3] Concerning the method itself, the fact must be emphasized once more that the technic is a very simple one, and on account of the absence of decolorization (as in Gram staining), the results are constant, when using the dye mixture at a constant pH-value. Such constancy of results is of course, the most essential part of staining at various definite pH-values. To this method, it is only necessary to have, (as we indicated previously), the standard dye mixture, $\frac{N}{100}$ HCl, $\frac{N}{100}$ NaOH, and the chart from which can be found the pH of each particular dilution.

To study the effect of the preliminary treatment, the same method of staining at various pH-values was used. For material, the kidney and liver of a freshly killed guinea pig were chosen. Immediately after removing from the body, these organs were transferred for 24 hours into the fixing fluids enumerated in Chart No. 2. After fixation, the pieces were washed in running water for 24 hours[4], were

[2]See footnote 1.

[3]As was reported the previous paper, the same method can be used for differential staining of bacteria.

[4]Except the pieces fixed in alcohol which were directly imbedded.

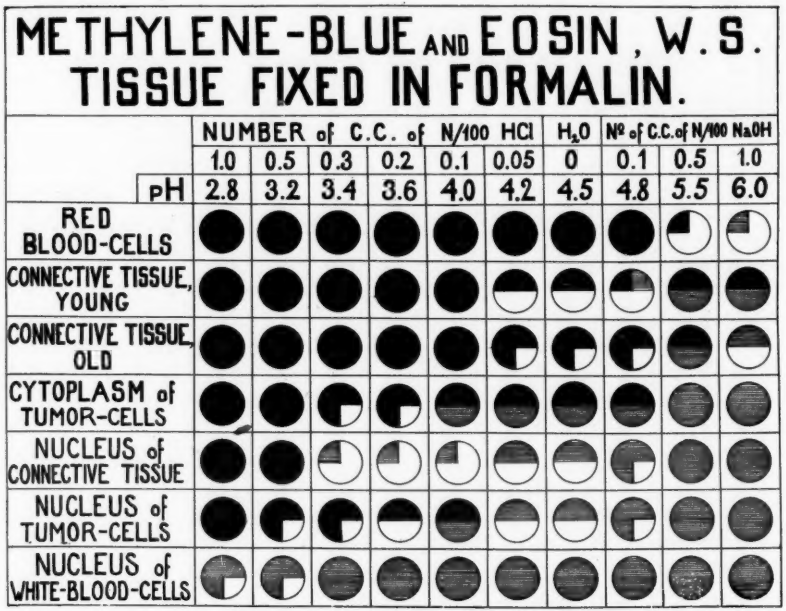

METHYLENE-BLUE AND EOSIN, W.S.
TISSUE FIXED IN FORMALIN.
NUMBER of C.C. of N/100 HCl
H2O
Nº of C.C. of N/100 NaOH
1.0 0.5 0.3 0.2 0.1 0.05 0 0.1 0.5 1.0
pH 2.8 3.2 3.4 3.6 4.0 4.2 4.5 4.8 5.5 6.0
RED BLOOD-CELLS
CONNECTIVE TISSUE, YOUNG
CONNECTIVE TISSUE, OLD
CYTOPLASM of TUMOR-CELLS
NUCLEUS of CONNECTIVE TISSUE
NUCLEUS of TUMOR-CELLS
NUCLEUS of WHITE-BLOOD-CELLS

CHART 2. RESULTS OF STAINING FORMALIN-FIXED TISSUE WITH THE EOSIN METHLYLENE-BLUE MIXTURE.

In the above circles: Depth of staining is indicated by the percentage of the circle which is shaded;

Staining by eosin indicated by solid shading; Staining by methylene blue indicated by barred shading;
Solid and barred shading in the same circle indicates equal staining by both eosin and methylene blue (i. e. purple).

Note that the nuclei of the various tissues begin to be stained by methylene blue at different pH-values, white blood cells at pH 2,8. connective tissue at pH 3.4, tumor cells at pH 4.0. The same is true of the cytoplasm of different tissues.

passed thru alcohol and imbedded in paraffin as usual. Before staining, the paraffin was removed by xylol. The slides were dried in the air and covered for 10 minutes with the dye mixtures at a definite pH-value. To secure the utmost uniformity in staining, the slides from different fixing fluids were stained by the mixture from the same test tube and at the same time. The dilutions of dye mixture with HCl or NaOH were always prepared immediately before staining. After staining, the slides were rinsed with distilled water, blotted by filter paper, dried in the air, dipped in pure xylol and covered with Canada balsam.[5]

To secure complete transparency the slides should be completely dried in the air. Introduction of this procedure is very important. Drying in the air does not destroy the fixed cells and in this way one can omit absolute alcohol and the oils usually used for this purpose. Omitting the alcohol is very essential, because decolorization of the slides in it is so quick that the data are rendered of little value.

As follows from the description of the technic, everything was the same in the various instances except for the use of different fixing fluids, i.e., if a difference in staining at the same pH-value is found, then this difference should be attributed only to one factor, i.e., to the stable effect of the fixing fluid.

The results of these experiments are presented on Chart 3.

At pH 4.0 we see that on the slides fixed in formalin everything is stained by methylene blue only. About the same results can be seen on a slide fixed in potassium ferrocyanide (K_4FeCn_6), where red blood cells alone show a slight purplish discoloration. With tungstic acid, one sees the presence of red staining in the cytoplasm and almost no blue tint in the red blood cells.

Entirely different pictures are given with the slides prepared from frozen sections and fixed in Zenker's fluid. On these slides everything is stained by eosin only.

An intermediate picture is shown by the slides fixed in methyl and ethyl alcohol, mercury bichloride, copper sulfate, picric acid and potassium bichromate, aluminium chloride. On these slides the red cells and cytoplasm are stained by eosin and the nuclei are stained by methylene blue.

Some of these findings can be explained from a chemical standpoint.

As is known, formalin forms inert compounds with amino groups of the amino acids of proteins and in this way it makes the proteins

[5] If desirable the slides can be left in xylol for a long time without any change in staining. The xylol was always left colorless after using the same jar for a couple of weeks.

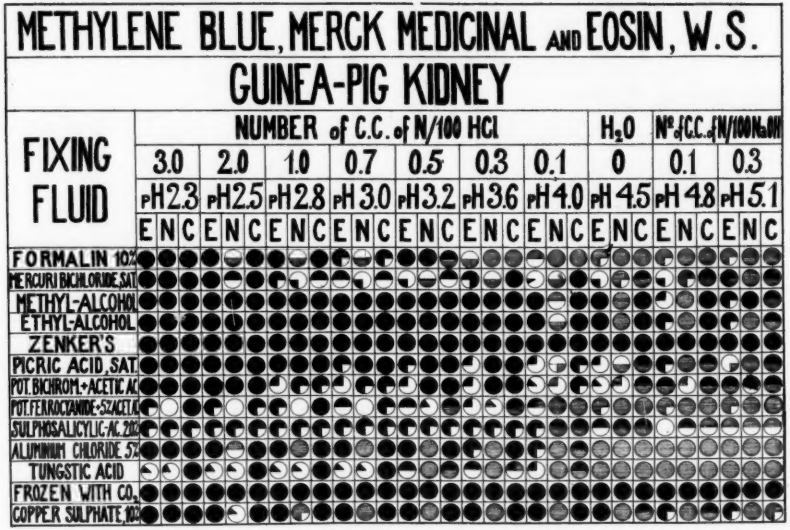


CHART 3. RESULTS OF STAINING WITH THE EOSIN-METHYLENE-BLUE MIXTURE AFTER VARIOUS FIXATIONS.

In the circles: Depth of shading is indicated by the percentage of the circle which is shaded;

Staining with eosin indicated by solid shading; Staining with methylene bluc indicated by barred shading;
Solid and barred shading in the same circle indicates equal staining by both eosin and methylene blue (i. e. purple).

The letters indicate as follows: E. red blood cells. N. nucleus of kidney cells. C. cytoplasm of kidney cells.

Note that after formalin, potassium ferrocyanide, tungstic acid, and aluminium chloride, all tissue elements are stained by methylene blue at much lower pH-values than in frozen sections and after fixation in Zenker's fluid, alcohols, and salts of heavy metals such as mercury and copper.

more acid or moves the isoelectric point of the proteins toward a lower pH-value. The same explanation can be applied to the action of the polyvalent acids.

The polyvalent cations as mercury, copper and aluminium combine with carboxyl groups of amino acids and in this way they make the proteins more alkaline or move the isoelectric point toward a higher pH-value.

It is interesting to note that in Zenker's fluid, the effects of mercury and chromium are added together and that to receive good differential staining we had to bring the dye mixture to about pH 8.9. Only at this reaction were we able to stain the nucleus by methylene blue.

At pH 4.8 the red blood cells are stained by methylene blue on slides fixed in the following fluids: formalin, potassium ferrocyanide, tungstic acid and potassium bichromate. With other fluids at this pH-value, the red cells are stained by eosin. These findings again show that formalin and polyvalent acids make the protein hemoglobin more acid, i. e., they move the isoelectric point toward a lower pH-value.

At a low pH (pH 2.8–3.0) the picture is different from the above described at pH 4 and 4.8. Here the nucleus is stained by methylene blue on slides fixed in formalin, aluminium chloride and copper sulfate. These findings do not agree very well with the simple chemical explanation given above.

If we compare the nuclear staining after fixation in formalin, mercury bichloride, aluminium chloride and copper sulfate, we find that at pH 3 in the case of slides fixed in mercury bichloride, the nuclei are stained by eosin; with the rest of the fluids (formalin, copper sulfate and aluminium chloride) they are stained by methylene blue.

We see that the effect of mercury in making the proteins more alkaline, is more pronounced than the effect of aluminium and copper at this low pH-range. It is probable that the aluminium and copper proteinate at these pH-values are dissociated, and for this reason their effect is lost.

It is important to note that we were able to get good differential staining with every fixing fluid; but at different reactions. For example, with formalin very finely differentiated slides were obtained at pH 3.2; with mercury bichloride at pH 5.1.

At the present time on account of the lack of information concerning the properties of the protein salts at different pH-values, it is impossible to give a detailed explanation of the above mentioned

facts. But it should be agreed that these findings show that we have a quite stable compound between the tissue proteins and the fixing fluids which change the staining properties of the tissues. The stability of these compounds is different at different pH-values.

To be able to stain the tissues equally with any fixing fluid, we should apply the dye mixture either at a very low pH-value (around pH 2.3) or at a very high pH-value (around 6–7).

Conclusions

The proposed method of differential staining at different reactions is sensitive enough to detect variation in staining properties or in chemical composition of the nucleus and cytoplasm of various tissues.

By the same method of staining, it is possible to detect the stable effect of different fixing fluids on the staining properties of the tissues.

These effects of fixing fluids in some cases can be explained from a simple chemical standpoint.

To study the staining properties of the different dyes they should be compared at the various definite pH-values and with definite fixing fluids.

To study the chemical composition or staining properties of different tissue elements, the dye mixtures should be used at definitely measured pH-values, and after definite fixing fluids.

STAINING CELL CONSTITUENTS IN DISEASED PLANT TISSUES

J. DUFRENOY[1]

Station Centrale de Phytopathologie et de Parasitologie, Paris, France

SUMMARY.—**Tissues of diseased plants, where embryonic cells, with dense cytoplasm, are to be studied cytologically in the same section with vacuolated cells, should best be killed with the Meves or Regaud fluids. The greatest changes in affected cells of diseased tissues are likely to occur in the vacuolar system, the contents of which should be well preserved in the killing process.**

Staining with acid fuchsin and decolorizing with light green, makes it possible, in properly killed tissues, to detect the slightest alteration in tissues, and to observe the different parts of the cell, nucleus, mitochondria and plastids, even when excessive vacuolation compresses the cytoplasmic inclusions.

The fact that structural differentiation in connection with physiological specialization in living tissues is frequently concomitant with changes in the staining reactions of the cell constituents makes cytological methods of investigation helpful to the physiologist and pathologist.

As the meristematic or embryonic cells of the growing points (stem tips, root tips) or of the cambial layers differentiate into the various elements of the older tissues of the plant, their dense cytoplasm becomes more and more conspicuously vacuolated. The maturing of the plant cell seems to be made evident chiefly thru the enlargement of the vacuoles, the cytoplasm meanwhile forming a thin parietal layer enclosing the nucleus or connected with the nucleus-bearing portion by slender strands.

The shape of the nucleus, which is relatively large in the undifferentiated cell, may become star-like as the rounded vacuoles press against the thin cytoplasmic layer around it. Many of the plastids, especially those containing starch grains, may also be so densely packed against the nucleus that its contour is obscured unless special staining methods be employed. As a rule, the various constituents in the cell become more difficult to demonstrate as the vacuoles enlarge and compact the other cytoplasmic inclusions.

[1]It is a pleasure to acknowledge the assistance kindly offered by Dr. L. W. Sharp in preparing this paper, as well as the opportunities for research work which were provided for the author, as a fellow of the International Education Board, in the Departments of Cytology and Plant Pathology at Cornell University.

When sections of living tissues are immersed in isotonic solutions of neutral red, methylene blue or brilliant cresyl blue the vacuolar material stains deeply, the reaction in many cases being due to the presence of phenolic compounds. The vacuolar material is liable to stain deeply in killed tissues also, obscuring the other parts of the cell. Good killing methods should therefore preserve the vacuolar material in such a way that it will not interfere with the demonstration of other cell elements.

The Regaud mixture (3% potassium bichromate, 4 parts; formalin, 1 part) generally preserves the tannoid contents of the vacuole in the form of a solid bright yellow mass, or of yellow drops. The nucleus, mitochondria and plastids may then be stained black with iron-alum hematoxylin; furthermore, the starch grains inside the plastids may be stained an amber yellow by immersing the sections in a KI+I solution after the mitochondria and plastids have been properly stained. But with iron-alum hematoxylin it is scarcely possible to obtain in the same section a proper differentiation of the structure in both meristematic cells (which require a longer time to destain) and older cells (which are more quickly destained by iron-alum). Staining with acid fuchsin, using the Kull method, makes it easy to obtain a good differentiation of nuclei and chondriome in all cells, both meristematic and highly differentiated. (The acid fuchsins obtained from the Empire Biochemical Co. and Coleman and Bell were found extremely satisfactory.) Staining embryonic cells as well as more highly differentiated cells is particularly important in the study of diseased tissues, since a very general pathological response is the formation of embryonic tissues at the margin of lesions by the normal meristematic tissues of the plant.

Altho very good results are obtained by staining tissues killed by the Regaud fluid, tissues which one intends to stain with acid fuchsin should preferably be killed with the Meves fluid (2% osmic acid solution, 1 part; 1% chromic acid solution, 5 parts). Poststaining with toluidin blue (0.5% aqueous solution) and destaining with aurantia (0.5% solution in 70% alcohol) will demonstrate the starch grains as blue inclusions within red chloroplasts or amyloplasts.

While in a few cases fungal haustoria may actually be observed entering cells without inducing changes in the staining reactions of the cell constituents (e.g., *Peronospora Schleidenii* in cells of onion bulbs), most parasites will bring about changes not only in the infected cells but also in neighboring uninfected cells. Induced changes such as would scarcely be evident morphologically may cause the nucleus and the plastids to stain more deeply with some dyes. When

morphological changes are evident, they will be accompanied by alterations in staining reaction.

Parasitic fungi or bacteria, as they infect a few cells of a tissue, induce metabolic changes in neighboring uninfected cells. These changes may involve a rejuvenating process, whereby a high metabolic rate is restored to some cells which return to the embryonic state. These cells may be serially arranged in a pathological cambium layer; this layer then yields two series of cells: (a) those formed on the side away from the infected tissue. Such cells by repeated division will form several layers of cells, each containing a large nucleus and dense cytoplasm with mitochondria, many of which will develop into plastids and form starch grains. (b) Cells formed on the side toward the infected tissue. These cells seem to age rapidly, as indicated by the development of huge vacuoles within them.

Enlargement of the vacuoles and storage of tannoid compounds within them are most significant features. They appear normally in epidermal cells and in some cells of the cambial layer, and they may be obtained pathologically at the margin of lesions caused by fungi or bacteria in resistant plants. Excessive vacuolation is also conspicuous in cells of affected tissues in mosaic-diseased plants.

Thus, by observing delicate staining reactions, the physiologist or pathologist may often be able to detect slight alterations in the tissues which would otherwise escape notice.

LITERATURE

CONN, H. J. 1925. Biological stains. Geneva, N. Y. (See pp. 62, 64, 69.)

GUILLIERMOND, A. 1927. Recherches sur l'appareil de Golgi et ses relations avec le vacuome. Arch. d'Anat. Micros. **23**, 1–98.

KRAUSE, R. 1927. Enzyklopädie der mikroskopischen Technik. Berlin. (See p. 1561.)

SHARP, L. W. 1926. Introduction to cytology. 2nd ed. New York. (See p. 112–122.)

Fig. 1–5. All material killed with Regaud fluid; all drawings made from the Zeiss apochromatic objective.

Fig. 1 and 2. Sections of tuber of potato affected by the Yellow Dwarf disease, showing cells near to the base of buds.

In the section illustrated in Fig. 1, the nuclei (N) mitochondria (m) and plastids were stained black with hematoxylin; the starch (S) in the amyloplastids was stained amber yellow with I and KI solution.

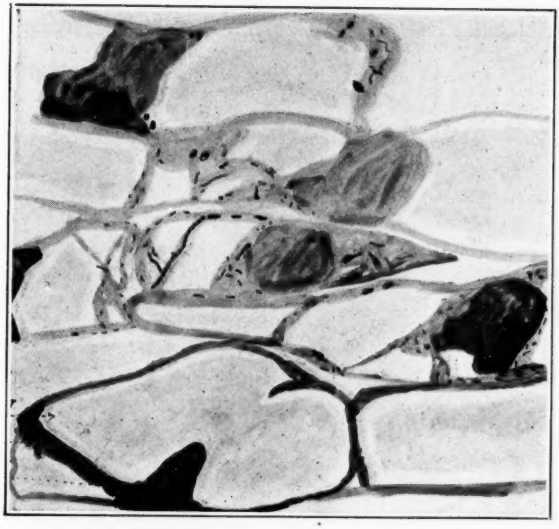
In the section illustrated in Fig. 2, the nuclei (N) and mitochondria (m) were stained a deep red with acid fuchsin, the cytoplasm (represented a deep grey) was colored light red, the vacuolar material V (represented a light grey) was stained yellow by the potassium bichromate in the Regaud killing fluid.

Fig. 3. Epidermal and subepidermal cells in longitudinal sections of leaf in bud of aster affected by "Aster Yellow". Nuclei (Ne) and mitochondria (m) and plastids (P) stained a solid black with hematoxylin; starch grains are represented a deep grey, as also 4 nuclei in deeper cells.

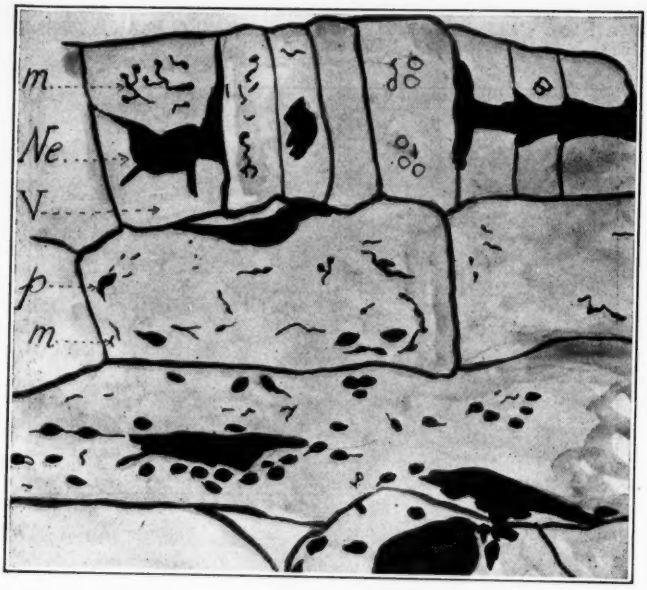
Fig. 4. Cells of medullary tissue, below growing point in "Stunted Dahlia." N, nuclei; m, mitochondria; V, vacuoles, the contents of which may show a reticulated appearance, or display a network pattern (G).

Fig. 5. Cell of Bean Pod, near to the margin of lesion caused by the fungus *Colletotrichum Lindemuthianum;* many mitochondria (m) are lined in linear series, as a result of active division; starch grains (s) are represented a deep grey, in the amytoplasts; vacuoles (V) show in the grey cytoplasm as empty canaliculi, representing the so-called "Holmgren canals."

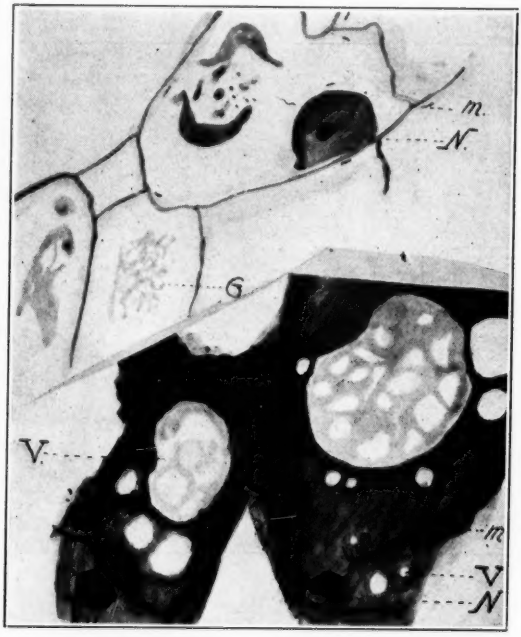
1



2



3



4

5

FURTHER STUDIES ON A MODIFICATION OF THE GRAM STAIN[1]

NICHOLAS KOPELOFF and PETER COHEN

Department of Bacteriology, Psychiatric Institute, Ward's Island, N. Y.

ABSTRACT.—**In perfecting the modification of the Gram-stain previously proposed, the following points are of interest:**

1. Acetone is too strong a decolorizer for Gram-positive organisms and alcohol too weak for Gram-negative organisms. Consequently, it is now recommended that equal parts of acetone (100% c.p.) and ethyl alcohol (95%) be used as a decolorizing agent. The time of application should not ordinarily exceed 10 seconds.

2. Aqueous basic fuchsin (0.1%) serves as a strongly contrasting counterstain. Prolonged application renders Gram-positive organisms doubtful or Gram-negative, while short application renders Gram-negative organisms doubtful or Gram-positive. Twenty (20) seconds is therefore recommended as the time of application of the counterstain.

3. The method here described, with due regard for its limitations, is of value in Gram-staining pure or mixed cultures as well as for organic materials, such as Acidophilus milk, feces, etc., either for research purposes or classroom use. The method is as follows:

Air-dry film and fix with least amount of heat necessary.

Flood with dye for 5 minutes. Previously mix 30 drops of a 1% *aqueous* solution of crystal violet or methyl violet 6B with 8 drops of a 5% solution of sodium bicarbonate. Allow the mixture to remain for 5 minutes or more.

Flush with iodine solution for 2 minutes. Two grams iodine dissolved in 10 cc. normal sodium hydroxide solution and 90 cc. water added.

Drain without blotting but do not allow film to dry.

Add a mixture of equal parts of acetone and alcohol drop by drop until the drippings are colorless. (10 seconds or less.)

Air-dry slide.

Counterstain for 20 seconds with 0.1% aqueous solution of basic fuchsin.

Wash off excess stain by short exposure to tap water and air-dry. If slide is not clear immersion in xylol is recommended.

In a previous paper (Kopeloff and Beerman, 1922) a description was given of a Gram stain modification which combined the principal features of methods devised by Atkins and Burke. Since our technic

[1]Presented before the Society of American Bacteriologists, Rochester, N. Y., December 28, 1927.

has found some favor at the hands of teachers and investigators, further attempts to improve it may now be recorded. Hucker and Conn (1927) have recently compared their ammonium oxalate method with that of Burke, as well as with our own technic and their conclusions must be of value to every bacteriologist, for they lay especial emphasis on the variability of the Gram reaction and the care in manipulation which is required to obtain satisfactory results. No method is fool-proof. Each investigator must apply the method best adapted to his requirements, keeping in mind the recent important contributions of Churchman (1927), Stearn and Stearn (1925), Burke and Ashenfelder (1926), and Conn (1927).

Our technic, as has been pointed out, is particularly valuable for the staining of organisms in organic materials, i.e., *Lactobacillus acidophilus* in milk or feces, etc.; for contrasting Gram-negative with Gram-positive organisms, as in impure cultures or mixtures of pure organisms; and finally for establishing Gram-variable organisms. The procedure is simple, lending itself readily to use in both class room and research laboratory.

There have been two causes for concern: first, in recommending the use of acetone there is danger of over-decolorization. Secondly, in using aqueous basic fuchsin as a counterstain there is danger of over- or under-staining. The present paper therefore deals with a clarification of these points.

The following organisms have been employed in this work: *Bacillus cereus*, Frankland identified by H. J. Conn; the fluorescent pathogen, known as *Pseudomonas pyocyanea* or *Ps. aeruginosa*, from J. C. Torrey; a culture from M. C. Kahn labelled "Micrococcus zymogenes" cultures of the colon and typhoid organisms and of *Proteus vulgaris* Hauser from the research laboratory of the Health Department of New York City; three cultures of non-pathogenic fluorescent rods from H. J. Conn, C. B. Coulter and J. C. Torrey respectively and a micrococcus No. 40a from H. J. Conn. The three cultures from H. J. Conn were the three used in the committee work on the Gram stain reported by him recently (1927 p. 81). Included with these was a culture of the lactic rod, *Lactobacillus acidophilus* (Moro) Holland identified by the authors.[2]

Slides were made of 18 to 24-hour nutrient broth cultures, pH 7.0, of each organism. Acetone c.p. and ethyl alcohol (95%) were employed as decolorizers separately and in combination, as follows: 100% acetone; 25% acetone+75% alcohol; 50% acetone+50% alcohol; 25% of acetone+75% alcohol; 100% alcohol. With each decolorizing solution the counterstain, aqueous basic fuchsin, 0.1% was applied

for 5, 10, 20 and 30 seconds respectively. All slides were prepared in triplicate. Averages only are presented in Table 1.

In glancing across Table 1 it will be seen that under each decolorizer is listed the number of seconds of application of the counterstain, i.e., 5, 10, 20 and 30 seconds. The bacteria have been divided into three groups: Gram-positive, Gram-variable and Gram-negative.

In the Gram-positive group are included *L. acidophilus* and *M. zymogenes*. It will be seen that in every instance under consideration both organisms proved to be Gram-positive. There are two trials with *L. acidophilus* listed, the first being with a 24-hour culture and the second with a 48-hour culture. As might be anticipated from experience with this organism which tends to become Gram-negative with age, the 24-hour culture yielded the more clear-cut positive results. In fact, there were only two instances in which the first trial with *L. acidophilus* failed to yield very strong positive results. It is noteworthy that both instances occurred when the counterstain was applied for 30 seconds—with 50% acetone+50% alcohol and 25% acetone+75% alcohol. In the second trial with a 48-hour culture of *L. acidophilus*, there were several instances in which very strong positive results were not obtainable, namely: at 5 and 20 seconds counterstain with 75% acetone+25% alcohol; at 20 and 30 seconds counterstain with 50% acetone+50% alcohol; and at 30 seconds counterstain with 25% acetone+75% alcohol.

Similarly, with the *M. zymogenes* culture there were several instances in which a very strong positive result was not obtained, notably at 10 and 30 seconds counterstain with 100% acetone; at 5, 10 and 20 seconds counterstain with 75% acetone+25% alcohol; and at 5 and 10 seconds counterstain with 50% acetone+50% alcohol.

The inferences from the data obtained with the above Gram-positive organisms are that acetone is perhaps too strong a decolorizer and that 30 seconds counterstain with 0.1% basic fuchsin is too long a period of time to obtain best results.

Turning now to the second group containing the variable organisms, *B. cereus* and coccus No. 40a, one finds that with only three exceptions there were no clear-cut positive or negative results. Ordinarily, *B. cereus* would not be considered a variable organism as regards its Gram-staining properties, but it must be so considered in the light of present results. It is of interest to note that with *B. cereus* the Gram-positive character is best brought out in the presence of alcohol as a decolorizer, rather than acetone. This corroborates the conclusions of Hucker and Conn using the same culture.

Considering the third group of Gram-negative organisms, one finds

TABLE 1. THE EFFECT OF ACETONE AND ALCOHOL, ALONE AND IN COMBINATION, ON THE MODIFIED GRAM STAIN TOGETHER WITH VARYING THE TIME OF APPLICATION OF THE COUNTERSTAIN (FUCHSIN).

Organism	100% Acetone				75% Acetone 25% Alcohol				50% Acetone 50% Alcohol				25% Acetone 75% Alcohol				100% Alcohol			
	Fuchsin—Sec.				Fuchsin—Sec.				Fuchsin—Sec.				Fuchsin—Sec.				Fuchsin—Sec.			
	5	10	20	30	5	10	20	30	5	10	20	30	5	10	20	30	5	10	20	30
Gram Positive																				
Lactobacillus acidophilus	++	++	++	++	++	++	++	++	++	++	++	+(+)	++	++	++	+(+)	++	++	++	++
Lactobacillus acidophilus	++	++	++	++	+(+)	++	+(+)	++	++	++	+(+)	+(+)	++	++	++	+(+)	++	++	++	++
Micrococcus zymogenes	++	+(+)	++	+(+)	+(+)	+(+)	+	++	+	+(+)	++	++	++	++	++	++	++	++	++	++
Gram Variable																				
Bacillus cereus	++	+	+(+)	+	+	+	+	+	+	+(+)	+	+	+	+	++	++	++	++	++	++
	—	—	—	—	—	—		—	—	—	—	—	—		?	?		—	?	?
Coccus No. 40a	+	—	+	—	—	—	—	—	—	—	—	—	—	—	—	+	+	—	—	+
	—	+	—	+	+	+	+	+	+	+	+	+	+	+	+	—	—	+	+	—
Gram Negative																				
Typhoid organism	——	——	——	——	——	——	——	——	—	——	——	——	——	——	—(—)	——	—	——	——	——
Colon organism	——	——	——	——	——	—	——	——	—	——	——	——	——	——	—(—)	——	∓	∓	—?	—?
Colon organism	——	——	——	——	—(—)	—	——	——	——	—	——	——	∓	——	——	——	∓	∓	∓	∓
Colon organism		——	——	——	——	——	——	——	—	——	——	——		——	—	——		——	?	?
Fluorescent pathogen (pyocyaneus type)	—	——	——	——	—	——	——	——	—	—	——	——	?	—	—	——	—(—)	∓	—(—)	?
Proteus vulgaris	—	—	——	——	—	——	——	——	—	—	—	——	——	?	—	——	—	∓	?	∓
Fluorescent rod (Coulter)	——	——	——	—	—(—)	——	—	——	——	—(—)	——	——	+	?	—(—)	——	∓	——	?	∓
Fluorescent rod (Torrey)	——	—(—)	——	——	——	——	——	——	——	——	——	——	——	——	——	——	∓	—	—(—)	—
Fluorescent rod (Conn)	?	—	——	—	——	—	—(—)	—(—)	?	?	—(—)	——	?	—	?	——	?	?	—	—

++ Very strong positive +(+) Strong positive + Fair positive ? Doubtful

∓ Negative predominating, and positive — Fair Negative —(—) Strong negative —— Very strong negative

exactly the converse of what is found with *B. cereus*, namely, that alcohol as a decolorizer is too weak to maintain uniformly very strong negative results. It will be observed that the greatest number of variable results with the Gram-negative organisms occurs with 100% alcohol and 75% alcohol+25% acetone.

The time of application of the counterstain indicates that 5 or 10 seconds is not as satisfactory as 20 or 30 seconds in obtaining very strong negative results in the third group of organisms.

The time of application of decolorizer thruout these tests deserves mention. Acetone decolorizes more rapidly than alcohol, therefore the former need not be applied for as long a time as the latter. In Table 2 will be found some relevant data:

TABLE 2.—TIME AND AMOUNT OF APPLICATION OF DECOLORIZER.

Decolorizer	Maximum Time of Application	Approximate No. of Drops
100% acetone	5 seconds	35
75% acetone+25% alcohol	7 "	45
50% acetone+50% alcohol	10 "	65
25% acetone+75% alcohol	15 "	95
100% alcohol	35 "	235

Previously, we (1922) have stated: "Add acetone drop by drop until no color is seen in the drippings from the slide which is slightly tilted. This usually requires less than 10 seconds and should be reduced to a minimum." The data in Table 2 refer to thin broth smears. Patently, thicker smears, or those containing more organic matter, require a somewhat longer period of time. But the important consideration is to reduce the time of application of the decolorizer to a minimum consistent with obtaining satisfactory results. Experience still remains the best teacher.

DISCUSSION

The Gram stain, no matter how it is modified, offers great difficulty in obtaining consistent results with different organisms. Such factors as the age of the culture, reaction, choice of decolorizer and counterstain, and time of application of each, influence the results. From the data here considered, it is apparent that acetone is perhaps too strong a decolorizer for Gram-positive organisms and alcohol is too weak for Gram-negative organisms. Ever ready to compromise in the face of conflicting tendencies, we now recommend the use of a 50–50 decolorizer, equal parts of acetone and alcohol. The time of application of the decolorizer is important and as Hucker and Conn (1927) state: "The

procedure of decolorizing is a very delicate one when used to distinguish between Gram-negative and Gram-positive organisms." For the decolorizer consisting of equal parts of acetone and alcohol a maximum application of 10 seconds is therefore recommended.

Aqueous basic fuchsin 0.1% is employed as a counterstain because it yields a striking contrast to crystal violet, or methyl violet 6B. But there is considerable danger in over-counterstaining Gram-positive organisms and under-staining Gram-negative organisms. It would appear from the results here compiled, that 20 seconds application of this counterstain will yield the most satisfactory results where different organisms are employed.

In their essential aspects our conclusions confirm those of Hucker and Conn (1927) and other important workers in this field. The phenomenon of the Gram stain is so complex that any simplified technic, such as is here attempted, cannot be offered didactically. Its limitations are as recognizable as its merits.

REFERENCES

BURKE, V., and ASHENFELDER, M. 1926. Notes on the Gram stain. Stain Techn. **1**, 63–67.

CHURCHMAN, J. W. 1927. Stability of the Gram reaction. Stain Techn. **2**, 21–23.

CONN, H. J. 1927. Variability of the Gram reaction. Report of the Committee, etc. Stain Techn. **2**, 80–87.

HUCKER, G. J., and CONN, H. J. 1927. Further studies on the methods of Gram staining. N. Y. Agr. Exp. Sta., Tech. Bull. 128.

KOPELOFF, N., and BEERMAN, P. 1922. The use of modified Gram stains. Jour. Inf. Dis., **31**, 480–482. *Also in* State Hosp. Quart. (N. Y.) **10**, 590–593. 1925.

STEARN, E. W., and STEARN, A. E. 1925. A study of the chemical differentiation of bacteria. J. Bact. **10**, 13–24.

DISCUSSION OF PAPER ON "METALLIC LAKES OF THE OXAZINES AS NUCLEAR STAIN SUBSTITUTES FOR HEMATOXYLIN"[1]

PERCY A. HOUSEMAN,

Camden, N. J.

The above named article in its opening paragraph appears to do some injustice to hematoxylin. The authors say "The exact chemical constitution (of hematoxylin) is not known.....The difficulty in obtaining a satisfactory hematoxylin.....is generally recognized. H. J. Conn has recently pointed out in this Journal the difficulties in the manufacture of hematoxylin to obtain a product sufficiently uniform to meet the requirements of histologists."

Later the authors say "The theoretical formulae for hematoxylin and hematein are given here for contrast."

The natural inference from these remarks is that hematoxylin is a very questionable individual, both in heredity and environment, and that serious consideration should be given to casting it out from the family of biological stains, in favor of anilin colors of purer breeding and more reliable conduct.

The enormous amount of experimental work which has been done on hematoxylin, and more particularly the elaborate and brilliant researches of W. H. Perkin and his pupils, have clearly established the constitution of hematoxylin, and render unwarranted the use of the word "theoretical" in connection with its constitutional formula.

In regard to "the difficulty in the manufacture of hematoxylin to obtain a product sufficiently uniform to meet the requirements of histologists," the authors fail to call attention to the fact that these difficulties have been overcome. H. J. Conn,[2] in the article referred to by the authors, after speaking of these difficulties, goes on to say "The new method.....turns out a more satisfactory hematoxylin. Solutions of it have been kept several months without apparent deterioration. The problem of hematoxylin seems therefore more nearly solved than ever before." During the year which has elapsed since the appearance of Dr. Conn's article, further experience has been obtained in the preparation of hematoxylin. The writer therefore believes that any claims which F. Proescher and A. S. Arkush make for metallic lakes of the oxazines must not rest at all on the statement that the constitution of hematoxylin is unknown, nor on the alleged difficulty of obtaining a satisfactory hematoxylin.

---

[1]Proescher, F., and Arkush, A. S. Stain Techn. **3**, 28–38. 1928.

[2]Conn, H. J. The hematoxylin problem. Stain Techn. **2**, 1–3. 1927.

NOTES ON TECHNIC

A NEW SUBSTITUTE FOR ETHYL ALCOHOL IN THE GRAM STAIN. It has been realized for some time that ethyl alcohol is not a perfect decolorizing agent in the Gram stain. The usual substitute is acetone; which, however is too rapid in its action. Kopeloff[1] now suggests a mixture of ethyl alcohol with acetone. This suggestion of Kopeloff's has been tried by the writer with good success. At the same time it must be pointed out that Scales[2] in a very similar technic uses a solution of safranin in a mixture of acetone and ethyl alcohol, this solution acting as decolorizing agent and counterstain at the same time. Scale's procedure is not really a modification of the Gram stain, altho it gives very similar results. It is possible to use his safranin solution as a combined decolorizing and counterstaining agent following the regular Gram procedure—i.e. gentian violet followed by iodine. This mixture of acetone and ethyl alcohol has the advantage as shown by Kopeloff of being slightly more rapid than the latter alone without being too swift in its decolorizing action.

One other objection to ethyl alcohol has been raised, however, which is not met by this substitute. The objection seems trivial but is nevertheless serious at certain institutions. There are, it seems, certain small institutions in which it is difficult to obtain and distribute to the students without violation of some prohibition ordinance. This does not apply in the case of research institutions or the larger teaching institutions; but bacteriology is coming to be taught more in high schools, and there seems to be a growing demand for a decolorizing agent for the Gram stain which cannot cause any legal difficulties in obtaining it and distributing it.

There are other alcohols besides ethanol which have decolorizing action, notably the methyl and the propyl alcohols. It has already been pointed out that the speed of decolorization by an alcohol varies inversely to its molecular weight. Thus methanol decolorizes more rapidly than ethanol and the propyl alcohols more slowly. Of these alcohols, methanol and isopropyl alcohol are commercially available and of course are not affected by the Volstead act.

In the writer's laboratory mixtures of methyl and isopropyl alcohol in various proportions have been compared in their speed of action in the Gram stain with ethyl alcohol alone. It has been found that a

---

[1]Kopeloff, N., and Cohen, P. Further studies on a modification of the Gram stain. Stain Techn. 3, 64. 1928.

[2]Scales, F. M. A new method for differential staining of bacteria. J. Inf. Dis. 31, 494-8. 1922.

mixture of equal parts of each comes the nearest to giving the same results as ethyl alcohol. If it is desired to obtain a slightly more rapid decolorizing agent, to give results like Kopeloff's mixture of acetone and ethanol, one may use a larger proportion of methanol in the mixture.

It is quite possible that some mixture of these two alcohols would give results in histological work comparable to those obtained with ethanol. This point has not been tried out. There does not seem to be the demand for a substitute for the latter in histological work that there has been in the Gram stain. There is no question but that the proposed substitute (50% ethyl and 50% isopropyl alcohol) is entirely satisfactory in the Gram technic.—H. J. Conn, Geneva, N. Y.